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Biochimica et Biophysica Acta 1459 (2000) 449–455

BIOCHIMICA ET BIOPHYSICA ACTA

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Fusion protein approach to improve the crystal quality of cytochrome bo_3 ubiquinol oxidase from *Escherichia coli*

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Abstract

Crystals of cytochrome bo_3 ubiquinol oxidase from *E. coli* diffract X-rays to 3.5 Å and the structure determination is in progress. The limiting factor to the elucidation of the structural detail is the quality of the crystals; the diffraction spots from the crystals are diffused which leads to difficulties in processing the data beyond 4.0 Å. Weak protein–protein contacts within the crystal lattice is assumed to be the cause of this problem. To improve these contacts, we have introduced protein Z to the C-terminal end of the subunit IV of cytochrome bo_3 and expressed both proteins as a single fusion. We have successfully obtained crystals of this fusion protein. The spot shape problem has clearly been solved in the crystals of the fusion protein although further optimization is necessary to obtain higher resolution. We also discuss the potential applications of this approach to the crystallization of membrane proteins in general.

Keywords: Ubiquinol oxidase; Cytochrome bo_3; Crystallization; Membrane protein; Fusion protein

1. Introduction

Heme–copper oxidases are integral membrane proteins located in the inner membrane of the mitochondria or the bacterial inner membrane. The oxidases are the final electron acceptors in the respiratory chain and catalyze the reduction of molecular oxygen to water. The terminal oxidases are divided into two variants based on substrate specificity: cytochrome *c* oxidases from mitochondria and many bacteria use cytochrome *c* as a substrate whereas some bacterial terminal oxidases utilize membrane-bound quinol as a substrate, and are thus called quinol oxidases.

Both cytochrome *c* and quinol oxidases have been extensively studied by biochemical and biophysical methods (for recent reviews see [1,2]). In recent years, there has been a great deal of progress in functional studies on cytochrome *c* oxidase, mainly due to the crystal structures [3–6]. Proton pumping mechanisms based on these structures have been extensively discussed, although no definitive conclusion has been obtained. In order to increase our understanding of this pumping mechanism and to study the ubiquinone binding site, we are studying cytochrome bo_3 ubiquinol oxidase from *Escherichia coli*. We have obtained crystals of cytochrome bo_3 [7] and structure determination is in progress (manuscript submitted). However, the work has been hampered by the qual-

Abbreviations: R_{merge}, $\Sigma|I_h-\langle I_h\rangle|/\Sigma I_h$ over all h, where I_h is the intensity of reflection h, respectively; PEG, polyethyleneglycol

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0005-2728/00/$ – see front matter
PII: S0005-2728(00)00183-3

ity of the crystals: the diffraction spots from the crystals show strong diffusion which causes difficulties in processing the data beyond 4.0 Å. The weak protein–protein contact in the crystal lattice is assumed to cause a disorder of the crystal leading to the diffused diffraction spots. Typically for membrane proteins, the cytochrome bo_3 crystal has a high solvent content (60%) due to the large detergent micelle surrounding the protein. Protein–protein contact in the membrane protein crystal lattice takes place mainly at the hydrophilic surfaces since the hydrophobic surfaces are covered by detergent micelles. This has proven to be the case for cytochrome bo_3 crystals [7].

In order to elucidate the precise details of the proton pumping mechanism of cytochrome bo_3, it is necessary to improve crystal quality. To achieve this aim, we investigated the possibility of increasing protein–protein interaction within the crystal lattice by extending the hydrophilic domain cytochrome bo_3. To obtain crystals of cytochrome *c* oxidase from *Paracoccus denitrificans*, an antibody F_V fragment was used for this purpose [8]. For our study, we generated a fusion protein of cytochrome bo_3 and protein Z. Protein Z, a modified form of the fragment B of protein A of *Staphylococcus aureus*, is a small, highly soluble and highly stable protein [9,10]. In addition, a high-resolution structure of the fragment B of protein A is available [11]. The protein Z was fused to the C-terminal, extracellular end of subunit IV of cytochrome bo_3 that is adjacent to the large solvent filled gap within the crystal lattice structure. The position of protein Z within the fusion protein is designed to improve the protein–protein contact in the crystals based on the packing of the wild-type cytochrome bo_3 crystals. In this paper, we present the crystallization and preliminary crystallographic studies of this fusion protein. We also discuss the potential applications of this approach to the crystallization of membrane proteins in general.

2. Materials and methods

2.1. Materials

Oligonucleotides were obtained from Scandinavian Gene Synthesis and Genset Oligos, France. All polymerase chain reactions (PCRs) were performed using Advantage Taq (Clontech) and dNTP (Boehringer Mannheim, Bromma, Sweden). PCR products were purified using the Qiagen gel purification kit (Qiagen, Hilden, Germany) and cloned into the pCR 2.1 TOPO vector (Invitrogen, Groningen, Netherlands). All constructs were sequenced using a 377 DNA sequencer (ABI) and the sequences analyzed using the program Vector NTI (Informax, Bethesda, MD, USA). Restriction endonucleases were obtained from Boehringer Mannheim and T4 DNA ligase from New England Biolabs (Hitchin, UK). DNA was prepared from *E. coli* cultures using plasmid miniprep kits from Qiagen or plasmid midiprep kits from Promega (Madison, WI, USA). The GasPack 100 anaerobic system and GasPack Plus envelopes were obtained from Becton Dickinson (Stockholm, Sweden). The peroxidase anti-peroxidase conjugate was purchased from Dako (Glostrup, Denmark) while nitrocellulose and the ECL detection kit were bought from Amersham Pharmacia Biotech (Uppsala, Sweden). Detergents were purchased from Anatrace (Maumee, OH, USA), Ni2+NTA resin from Qiagen and the MonoQ column from Amersham Pharmacia Biotech.

2.2. Insertion of unique restriction sites to the C-terminal of subunit IV

The pMB908 (pJRHisA) was a gift from Professor R. Gennis, University of Illinois, Urbana, IL, USA. This construct consists of pBR322 into which the whole cytochrome bo_3 operon has been cloned, including a His9-tag at the end of cyoA, yielding a plasmid of approximately 10 kb in size. The addition of unique restriction sites (MCS) to the 3′ end of subunit IV in pMB908 was performed by the PCR method of splicing by overlap extension as previously described [12]. Briefly, the method entails the use of four different primers, two encompassing the entire sequence to be changed (5′-TTCACGAACGTGATGCATTCTGGGTTTTGA-3′ and 5′- CTCCTTGCATGCGCAAGCCGCATACGGTCC-3′), one on either end of the new construct, and two centrally positioned primers (5′-GCACAGCGGCCGCGAGCTCACGCGTATGTCTAGATAAGAGCGGCGGTTATG-3′ and 5′-ATCTAGACATACGCGTGAGCTCGCGGCCGCTGTGCATCACATGTTGTA-

3′) containing the sequence for the restriction sites (*Not*I, *Sac*I, *Mlu*I, *Xba*I) giving rise to two fragments containing overlapping sequences. These two fragments were then used as template in order to amplify the full-length 2.4 kb fragment incorporating the MCS. This was then ligated into pMB908 generating pMB930.

2.3. Generation of cytochrome bo_3+protein Z fusion protein

Protein Z was amplified from the commercially available vector pEZZ-18 (Amersham Pharmacia Biotech) using oligonucleotide sequences (5′- GCGGCCGCGACAACAAATTCAACAAAGAACAACAAAAT-3′ and 5′-TGATGCTCAGGCGCCGAAAGTAACGCGT-3′) which code for a *Not*I site at the 5′ end and *Mlu*I site at the 3′ end, respectively. The PCR fragment was cloned into pMB930 to yield the vector construct pMB1048.

2.4. Expression of cytochrome bo_3+protein Z fusion protein

The DNA coding for cytochrome bo_3+protein Z fusion was transformed into a terminal oxidase deficient *E. coli* strain, GO105 [13], which will only grow under aerobic conditions after introduction of a functional cytochrome bo_3. The GO105 cells were grown anaerobically in a BBL GasPack 100 anaerobic system using GasPack Plus envelopes and made competent using standard methods [14]. Colonies obtained were used to inoculate 10 ml of LB supplemented with ampicillin which were incubated overnight at 37°C. The specific expression of subunit IV+Protein Z was assessed by Western blot analysis using a method based on the one described by Rondahl et al. [15] using peroxidase anti-peroxidase as a substrate. For the large-scale expression, GO105 cells expressing the fusion protein were grown in a 10-liter fermentor (OD_{550} = 3.0), according to the method described previously [16].

2.5. Purification and crystallization of cytochrome bo_3+protein Z

Membrane preparation and protein purification was performed as described previously [7]. Crystals for this fusion protein were obtained by the hanging-drop vapor diffusion technique under similar conditions to the wild-type cytochrome bo_3 [7]. The enzyme solution contained 20 mM Tris–HCl buffer (pH 7.5) with 1% octylglucoside and 20 mg/ml protein. A reservoir solution of 7–8% (w/v) PEG 1500, 100 mM NaCl, 100 mM $MgCl_2$, 100 mM Hepes–NaOH (pH 7.0) and 5% ethanol was used. The protein solution was mixed in a 1:1 ratio with the reservoir solution and left to equilibrate at 10°C.

2.6. Data collection and processing

Data was collected from frozen crystals. Prior to freezing the crystals in liquid nitrogen they were soaked in cryo-solutions in which both the glycerol and PEG 1500 concentrations were gradually increased. Data was collected at the ESRF (European Synchrotron Radiation Facility) on beamline ID14/EH4. Data collection was performed at 100 K. Image data were processed by the HKL program suite [17].

3. Results

3.1. Expression of cytochrome bo_3+protein Z

We were able to assess the expression of cytochrome bo_3+MCS and cytochrome bo_3+protein Z in two ways. Firstly, transforming the modified forms of cytochrome bo_3 into the terminal oxidase deficient strain, GO105, gave colonies which grew under exactly the same conditions as those transformed with wild type bo_3. Expression of the cytochrome bo_3+MCS construct was further assessed by western blot analysis using an anti-His antibody directed against the His tag at the C-terminal end of subunit II. This construct yielded a strong and specific band corresponding to subunit II (33 kDa) which was identical to that seen for the native bo_3 control (Fig. 1A). Western blot analysis of cytochrome bo_3+protein Z fusion probed with peroxidase anti-peroxidase which binds to protein Z, yielded a strong and highly specific band of approximately 21 kDa which was not present in the negative control of cytochrome bo_3 only (Fig. 1B). Large-scale expression of cytochrome bo_3+protein Z construct was per-

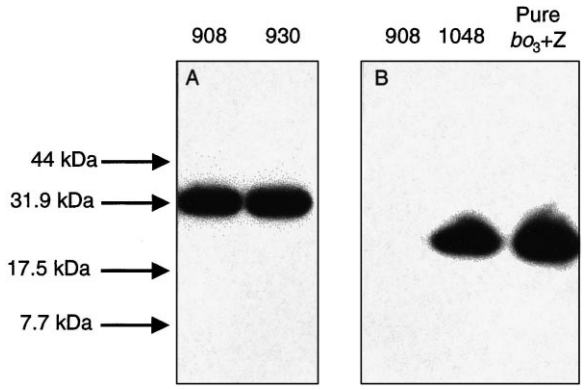

Fig. 1. Western blot analysis of membranes prepared from GO105 cells expressing wild-type and modified forms of cytochrome bo_3. Blot A was probed with an anti-His antibody specific to the His tag sequence at the C-terminal end of subunit II and shows a specific band corresponding to subunit II for both the wild-type form of cytochrome bo_3, 908, and the cytochrome bo_3 with the MCS, 930. Blot B was probed with peroxidase anti-peroxidase and shows a clear band corresponding to subunit IV+protein Z for 1048 which is not present for 908. This blot also shows the specific band obtained for purified 1048 diluted 1:20 000.

formed over about 8 h and showed a similar growth pattern to the native cytochrome bo_3.

3.2. *Purification and crystallization of cytochrome bo_3+protein Z*

Cytochrome bo_3+protein Z fusion protein was purified under the same conditions as the wild-type enzyme [7]. Both 'low' and 'high' imidazole fractions yielded crystals under similar conditions to the wild-type enzyme. However, the crystals of the fusion protein were obtained over a wider pH range (6–8) than the wild type's (pH 7–7.5). The external appearance of the crystals for both 'low' and 'high' imidazole fractions were almost identical but clearly different from that of the wild-type cytochrome bo_3 (Fig. 2). The crystals of the fusion protein have a more regular shape than the wild-type crystals. The shape of the diffraction spots from the fusion protein have been significantly improved; however the resolution of the crystals has been decreased from the wild-type crystals (Fig. 3). We have collected a data set from a crystal of the fusion protein which diffract X-rays up to 6 Å. The crystal belong to the space group $P2_1$ with the cell dimensions of $a = 93.4$ Å, $b = 328.7$ Å, $c = 131.1$ Å and $\beta^* = 92.1°$. The data set was processed to 6 Å resolution with an overall completeness of 79% (69% in the outer shell) using 26 844 reflections (15 665 unique). The R_{merge} values in the lowest (100–11.9 Å) and highest (6.3–6.0 Å) resolution shells are 0.029 and 0.45, respectively with the overall R_{merge} value being 0.045. Assuming a tetramer of cytochrome bo_3 ($M_r = 576$ kDa) per asymmetric unit, the ratio of the volume to unit protein mass (V_M) and the solvent content of a crystal are 3.5 Å^3/Da and 60%, respectively. The space group of the crystals are different from the wild-type crystals. These crystals belong to the space group $C222_1$ with the cell dimensions of $a = 91.3$ Å, $b = 370.3$ Å and $c = 232.4$ Å; the V_M and solvent contents are 3.4 Å^3/Da and 59%, respectively. Some of the cell parameters and solvent contents of the fusion protein

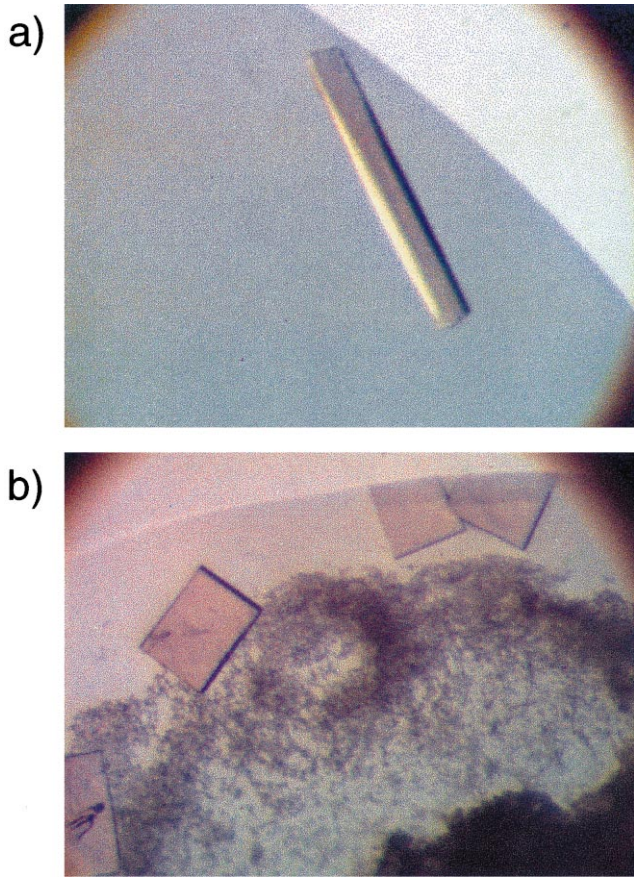

Fig. 2. Crystals of cytochrome bo_3 ubiquinol oxidase from *E. coli*. (a) A wild-type crystal. The size of the crystal is of $0.6 \times 0.2 \times 0.1$ mm. (b) Crystals for cytochrome bo_3+protein Z fusion. The size of the largest crystal is $0.3 \times 0.4 \times 0.1$ mm.

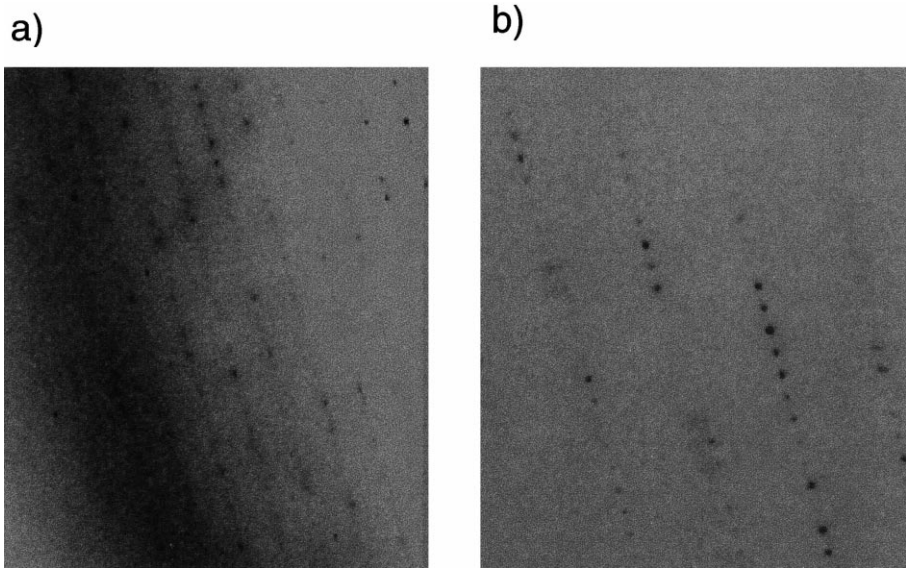

Fig. 3. X-Ray diffraction patterns of (a) a wild-type crystal around 4 Å resolution and (b) a crystal of cytochrome bo_3+protein Z fusion around 6 Å. Both data were collected at the beam line ID14/EH4 of ESRF. Exposure time and oscillation range are 5 s and 0.3°, respectively.

crystals are similar to those for the wild type. As mentioned above, the conditions under which crystals were obtained for the fusion protein were also similar to those for the wild-type cytochrome bo_3. This indicates that the packing of the wild-type and fusion protein crystals could be related. Unfortunately molecular replacement trials have not been successful so far, since the new crystal form has four fusion proteins in the asymmetric unit, compared with two for the wild-type, and the data resolution is limited.

4. Discussion

The crystal packing of the wild-type cytochrome bo_3 has been determined by molecular replacement; the lattice has been shown to contain large solvent filled gaps adjacent to the C-terminal end of subunit IV (Fig. 4). This space is a partly filled with solvent and partly filled with the detergent micelle around the protein. In an attempt to increase protein–protein contact in the cytochrome bo_3 crystals, we fused protein Z to the C-terminal end of subunit IV of cytochrome bo_3. The C-terminus of subunit IV is positioned on the periplasmic side of the cell; thus, protein Z, which is readily secreted and stable, was an ideal fusion partner. The fusion protein was designed in such a way as to place protein Z in the solvent filled gap, thus allowing it to make a contact with the next cytochrome bo_3 in the crystal lattice (Fig. 4). The fusion protein expressed readily in an *E. coli* deletion strain demonstrating that the presence of the Protein Z had not affected the expression, folding or function of cytochrome bo_3. In addition the growth characteristics of the fusion protein under large-scale expression conditions was similar to the wild-type cytochrome bo_3 although the overall expression was slightly increased. Similar to the wild-type cytochrome bo_3, the purification of the fusion protein on the Ni-NTA column yielded high and low imidazole peaks [7]. It is thought that partial and specific proteolysis of the C-terminus of the subunit II including His9 is responsible for the elution of the first protein peak at such low concentrations of imidazole. However, in contrast to the wild-type cytochrome bo_3, cytochrome bo_3+protein Z consistently yielded crystals for both the high and low imidazole protein fractions.

In principle, the method of developing a fusion protein worked; crystals of the fusion protein were obtained under very similar conditions to the wild-type cytochrome bo_3. We were able to achieve our first objective; the diffraction spots do not exhibit the

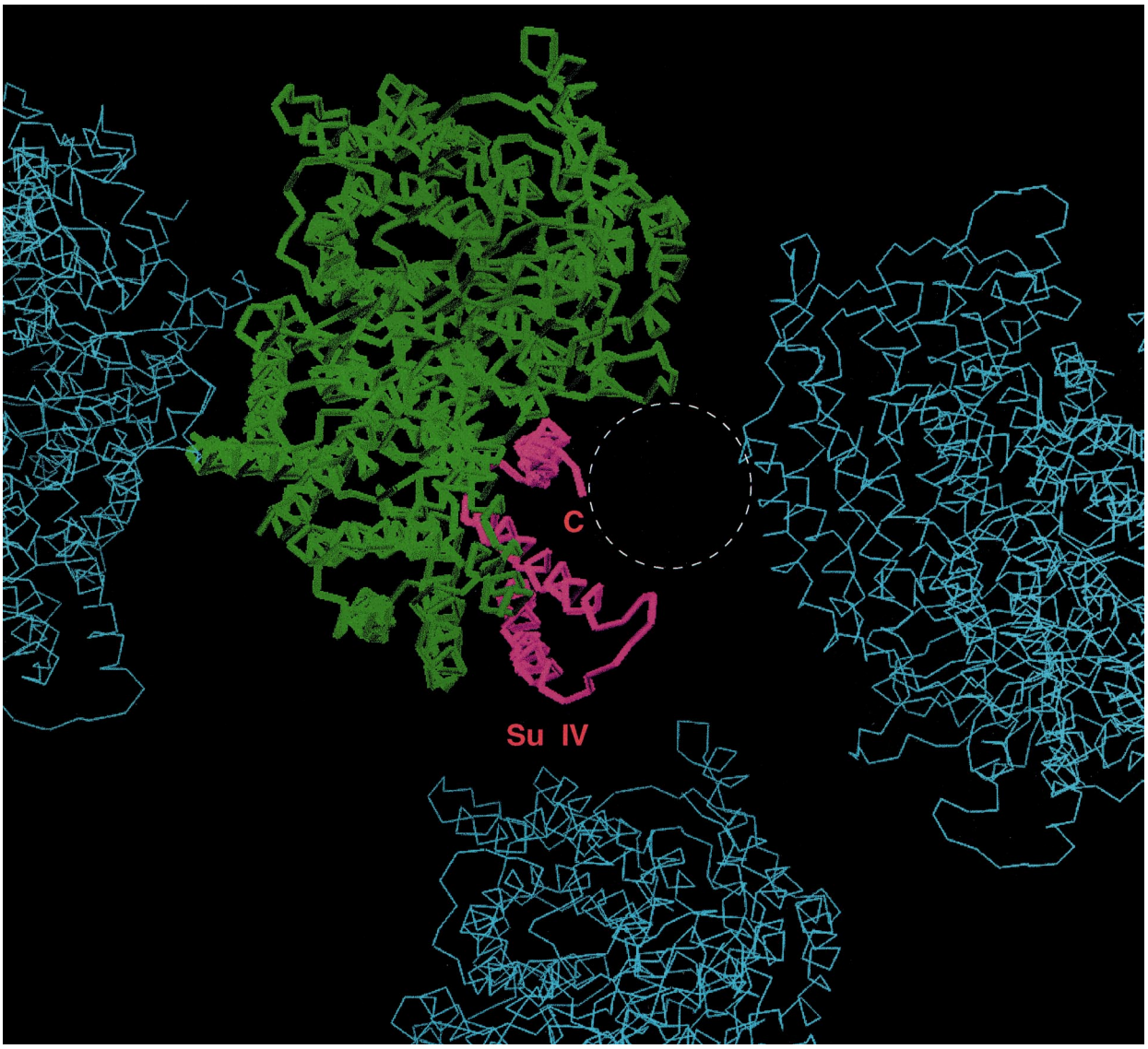

Fig. 4. Packing of the wild-type cytochrome bo_3 crystal. One molecule of cytochrome bo_3 is highlighted using thick lines. The subunit IV and other subunits of the highlighted molecule are in magenta and in green, respectively. The position of the C-terminus of the subunit IV is labeled as 'C'. All other symmetrically related molecules are drawn using light-blue thin lines. The dotted circle shows the approximate size and position of the protein Z when it is fused at the C-terminus of the subunit IV. The figure was produced using the program O [18].

diffusion seen for the wild-type crystals (Fig. 3). However, two unexpected things happened; the space group for the fusion protein crystals is different, although it appears to be related to that of the wild-type crystals and the fusion-protein crystals exhibit decreased resolution compared with the wild-type crystals. The change of the space group should be due to the change of the protein–protein contact of the crystal caused by introduction of protein Z. On the other hand, this clearly indicates that the protein Z is, indeed, involved in the protein–protein contact in the lattice. The loss of the resolution could also be related to the rearrangement of the packing. It is known that detergent selection is very important in order to obtain well diffracting membrane protein crystals; the detergent whose micelles fit best into the solvent gap within the crystal lattice is assumed to give the best crystals. Since the shape of the solvent gap is altered by the presence of the protein Z, the optimal detergent may be different for crystals of the

fusion protein. We are currently screening to identify the detergent that will yield crystals that diffract to the highest possible resolution. In addition, we are also experimenting with alternative fusion partners

The solvent-filled gaps within membrane protein crystals are quite large. The gap in the cytochrome bo_3 crystal lattice is as large as 100 kDa. Thus, it may be possible to apply this principle to the fusion of other larger protein molecules to the C-terminus of subunit IV. This method could be useful not only for improving the quality of the membrane protein crystals but for the effective crystallization of other proteins including integral membrane proteins. The limitation is that the protein of interest must express in *E. coli* with its N-terminus on the extracellular side. In spite of this limitation, the approach could be applied to some interesting proteins including the G-protein coupled receptors. We are currently investigating this possibility.

Acknowledgements

This work was supported by the EU-Biotechnology Program and the Swedish Research Councils, NFR. Data collection was performed at ESRF ID14/EH4 under the proposal LS1609. We acknowledge the help of W. Burmeister for the data collection.

References

[1] M. Wikström, Biochemistry 39 (2000) 3515–3519.
[2] S. Iwata, J. Biochem. 123 (1998) 369–375.
[3] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Nature 376 (1995) 660–669.
[4] C. Ostermeier, A. Harrenga, U. Ermler, M. Hartmut, Proc. Natl. Acad. Sci. USA 94 (1997) 10547–10553.
[5] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Science 272 (1996) 1125–1136.
[6] S. Yoshikawa, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, E. Yamashita, N. Inoue, M. Yao, M.J. Fei, C.P. Libei, T. Mizushima, H. Yamaguchi, T. Tomizaki, T. Tsukihara, Science 280 (1986) 1723–1729.
[7] J. Abramson, G. Larsson, B. Byrne, A. Puustinen, A. Garcia-Horsman, S. Iwata, Acta Crystallogr. D 56 (2000) in press.
[8] C. Ostermeier, S. Iwata, B. Ludwig, H. Michel, Nat. Struct. Biol. 2 (1995) 842–846.
[9] B. Nilsson, G. Forsberg, T. Miks, M. Hartmanis, M. Uhlen, Curr. Opin. Struct. Biol. 2 (1992) 569–575.
[10] E.R. LaVallie, J.M. McCoy, Curr. Opin. Biotechnol. 6 (1995) 501–506.
[11] J. Desienhofer, Biochemistry 20 (1981) 2361–2370.
[12] J.N. Rumbley, E.F. Nickels, R.B. Gennis, Biochim. Biophys. Acta 1340 (1997) 131–142.
[13] T.M. Kaysser, J.B. Ghaim, C. Georgiou, R.B. Gennis, Biochemistry 34 (1995) 13491–13501.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis,Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1987.
[15] L. Rondahl, B. Nilsson, E. Holmgren, J. Biotechnol. 25 (1992) 269–287.
[16] C.D. Georgiou, P. Cokic, K. Carter, D.A. Webster, R.B. Gennis, Biochim. Biophys. Acta 933 (1988) 179–183.
[17] Z. Otwinoski, W. Minor, Methods Enzymol. 276 (1997) 307–326.
[18] T.A. Jones, J.-Y. Zou, S.W. Cowtan, M. Kjeldgaard, Acta Crystallogr. A 47 (1991) 110–119.